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Testing endosymbiont cellular organelles and compounds identifiable therewith

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Testing endosymbiont cellular organelles and compounds identifiable therewith.

The invention relates to diagnosis of disease and/or determination of functioning of cellular organisms, being of multi-cellular or unicellular nature, being visible by the naked eye or being a micro-organism.

A diagnostician of disease studying (mal)functioning of cellular
5 organisms, classically can employ a broad range of inroads into said organism to obtain relevant information as to the various aspects of said malfunctioning. These inroads vary widely, examples range from detecting relative ratios of kidney stones by studying urinary samples obtained from various patients, from probing for the presence or absence of intestinal ulcers via endoscopy,
10 from scanning for detectable tumors by nuclear magnetic resonance (NMR), from detecting diabetes by testing for insulin levels and/or glucose concentration in blood plasma, to determining cancer proneness by determining transcriptional levels of oncogenes, and so on.

Currently, detection of disease or malfunctioning (or vice versa, of
15 health and proper functioning) of higher organisms, such as animals and plants relies very much on testing samples obtained from these organisms and studying these samples in a laboratory. Often when a fruitful method capable of determining, identifying or detecting (aspects of) a disease or malfunctioning of an organism has been found it is in general also useful in testing or
20 screening for compounds or methods useful for treatment of (aspects of) said disease or malfunctioning or useful in testing or screening for compounds or methods involved in causing (aspects of) said disease or malfunctioning; by using the same or a similar method as used in diagnosis it is generally possible to generate an assessment of the usefulness of such candidate compounds or
25 methods in treating and/or causing the disease or malfunctioning in question. Clearly, life science laboratories are always in the need of yet other inroads into organisms to obtain yet more information relating to disease or

malfunctioning and to compounds and methods related to cause and/or treatment of disease or malfunctioning.

5 The invention provides a method for determining (mal)functioning of a cellular organism comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in relation to another nucleic acid or gene product present in a sample obtained from said organism. Endosymbiont cellular organelles are those organelles of a eukaryotic cell that are thought to have been derived of prokaryotic bacteria
10 very early on in the evolution of eukaryotic cells; these bacteria (is thought) have engaged in a symbiosis with early eukaryotic cells, and at present, eukaryotic cells comprising these endosymbiont organelles in general cannot live without them; none of the present eukaryotic cells would function properly without mitochondria, and most plant cells would at least considered to be
15 malfunctioning when no proplastids, or organelles derived thereof, such as chloroplasts, etioplasts, amyloplasts, elaioplasts or chromoplasts were present. These organelles in general appear to be at least partially self-replicating bodies which, although under some nuclear controls, still possess considerable autonomy.

20 In particular, the invention provides a method whereby said relative ratio of an endosymbiont cellular organelle nucleic acid and/or gene product thereof is determined in relation to the amount of essentially nuclear nucleic acid detectable in said sample (be it DNA or RNA), or in relation to gene products (derivable by transcription and/or translation, such as mRNA or
25 (poly)peptides) of said nuclear nucleic acid, (nuclear nucleic acid herein comprises chromosomal DNA and the RNA transcribed therefrom) for example present in nuclear or cytoplasmatic fractions or parts of said sample. DNA or corresponding mRNA encoding components of small nuclear ribonucleoprotein (SNRNP), or other essentially common nucleic acid derived from chromosomal
30 DNA, is particularly useful to test, because of its ubiquitous presence. In this

way, the invention provides a method for studying for example mitochondrial and/or proplastid related disease. Endosymbiont cellular organelle related disease encoded by defects in said organelle's DNA manifests in many different syndromes and is often variable in its expression (and thus in general hard to
5 detect by testing for clinical parameters alone) due to heteroplasmy, whereby mutant and wild type nucleic acid can be found in one cell, whereby its distribution can vary. Endosymbiont cellular organelle related disease is often aggravated with increasing age of the affected individual. Endosymbiont cellular organelle related disease can also often be observed after treatment
10 against other disease with various drugs, and then contributes to various side-effects of those drugs that basically one would like to avoid during treatment. Those side effects can now be better studied by using a method as provided herein.

Furthermore, the invention provides a method whereby said relative
15 ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof is determined in relation to the amount of a distinct endosymbiont cellular organelle nucleic acid detectable in said sample (be it DNA or RNA), or in relation to gene products (derivable by transcription and/or translation, such as mRNA or (poly)peptides) of said endosymbiont cellular organelle
20 nucleic acid. In one aspect of the invention the method involves determining a ratio between organelle DNA, such as mtDNA, and the corresponding transcriptionally derivable organelle RNA, in the example the related mtRNA, or translated gene product. In yet another aspect of the invention, the ratio between two distinct organelle DNA's or related gene products is determined.

25 Other possible uses of the invention lay in candidate drug testing, for beneficial activity and/or side effects of possible medicaments or pharmaceutical compositions such as candidate anti-parasitic compounds, antibiotic compounds, cytostatic compounds, and so on. For example, the invention provides a method for determining therapeutic activity and/or
30 possible side-effects of a candidate compound, for example in determining its

usefulness for treatment of malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism, preferably said organism or an essentially related organism, such as belonging to the same species or genus, having been provided with said compound. In terms of the invention, therapeutic activity means the capability of at least in part treating a disease. In one embodiment of the invention, said therapeutic activity comprises a therapeutic activity against an HIV-related disease. By a side effect of a compound is meant herein another effect than the purpose of said compound. Said side-effect may be an unwanted effect. For instance, a therapeutic compound may counteract a disease and simultaneously reduce the metabolism of an organism. Said reduction of said metabolism is then referred to as a (negative) side-effect. Alternatively, a side-effect of a compound may be a beneficial effect, like for instance immunity against yet another disease.

Also use for (selective) toxin testing, of e.g. herbicides, insecticides, anti-parasitic compounds, antibiotic compounds is provided herein. The invention provides a method for determining toxic activity of a candidate compound, for example in determining its usefulness for causing malfunctioning of a cellular organism, e.g. by having a cytostatic or even cytotoxic effect, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism, preferably said organism or related organism, having been provided with said compound.

In a preferred embodiment, selectivity is also tested, using or applying the method as provided herein (preferably in parallel experiments) on or to a first organism and on or to an essentially unrelated second organism, if desired belonging to a different family or order, but preferably belonging to at least a different class or phylum, most preferably belonging to a different kingdom of organisms. Selectivity aspects are for example tested by testing the

compounds in (if desired only in cells of) a first target organism (such as a bacterium or parasite) as well as of testing the host or cells thereof, being an essentially unrelated second organism, for example a mammal or plant, or by testing of a crop plant or cells thereof as well as testing an essentially
5 unrelated weed plant or cells thereof with said compound, to determine for example selective toxic or selective therapeutic effects. It is also provided to test normal cells derived from an individual in parallel or comparison with aberrant cells, such as tumour cells derived from the same individual, to detect or screen for a tumour-specific or at least selective cytostatic or cytotoxic
10 compound for use in therapy of said individual or others with similar or related disease.

With a method of the invention, a relative ratio is for instance determined by measuring the amount of said nucleic acid(s) and/or gene
15 product(s) present in said sample, usually after at least one processing step, like for instance amplification of target nucleic acid. After said amounts have been measured, said relative ratio can be determined by dividing one amount by another.

Minute amounts of target nucleic acid can be detected and quantified by
20 using enzymatic amplification. Examples of enzymatic amplification techniques are a polymerase chain reaction (PCR)¹, nucleic acid sequence-based amplification (NASBA)², SDA, TMA, and others. Specific amplification of a target nucleic acid sequence can be achieved by adding two primer sequences to a reaction. An amplified region can be detected at the end of an
25 amplification reaction by probes that are specific for said amplified region. Alternatively, an amplified region can be detected during generation of said amplified nucleic acid in said amplification reaction³. In the latter protocol a signal of a label attached to a probe can become detectable after said probe has hybridised to a complementary nucleic acid. Examples of such probes that

enable real-time homogenous detection in amplification reactions are TaqMan³ and Molecular Beacon probes^{4,5}.

Quantification of a target nucleic acid sequence is commonly accomplished by adding a competitor molecule, which is amplified using the same primers and which contains sequences that allow discrimination between competitor and target nucleic acid sequence^{2,6}. The ratio between amplified competitor and target nucleic acid sequence can be used to quantify said target nucleic acid sequence. Detection of competitor or target nucleic acid sequence can for instance be achieved at the end of the amplification reaction by probes that are specific for said amplified region of competitor or target nucleic acid sequence or during generation of said amplified nucleic acid in the amplification reaction. In the latter protocol a signal of a label attached to a probe can become detectable after said probe has hybridised to a complementary target nucleic acid and when said target has exceeded a threshold level; the time or cycle number to positivity. In other methods for quantification, the time to positivity can be used for quantification without addition of a competitor⁷.

A method of the invention is very suitable for, among others, determining (mal)functioning of a cellular organism, candidate drug testing and selective toxin testing. Many reactions have been carried out using a method of the invention, which has proven to be a useful tool (see examples). An even more precise result can be obtained using a method of the invention when double spreading in the result is avoided. Generally, double spreading in the result of a method of the invention is obtained due to varieties in conditions in different reaction mixtures. For instance, to be able to detect and quantify specific nucleic acids present in a sample, an amplification step is often necessary. However, the temperature of the reaction mixture of nucleic acid 1 may be slightly higher than the temperature of the reaction mixture of nucleic acid 2. This may result in a higher yield of nucleic acid 1 and, hence, in

a higher ratio of the amount of nucleic acid 1 versus nucleic acid 2 than would have been obtained if the temperature of reaction mixture 1 had been exactly the same as the temperature of reaction mixture 2. Because of said temperature difference in said reaction mixtures, the determined ratio is not exactly the same as the real ratio of the two nucleic acids present in the initial sample. Likewise, minute variations in other conditions like for instance the amount of enzyme added can lead to variations in the determined amounts of nucleic acids 1 and 2. Thus, the measured amounts of nucleic acids 1 and 2 may vary independently from each other. Independent variations in said determined amounts may result in an even larger variation in the calculated ratio of said measured amounts. This is called the double spreading in the result. Thus, by double spreading is meant herein at least one variation in an obtained result, due to a variety of at least one reaction condition in at least two reaction mixtures. For instance, also the total amount of volume may differ slightly between two reaction mixtures.

In some particular cases, double spreading in a result may exceed the variations of the relative ratio of an endosymbiont cellular organelle nucleic acid and/or gene product thereof in an organism which is due to a certain disease or treatment. For instance, inhibitors of viral polymerase are often used for treatment of HIV. Inhibitors of viral polymerase may also affect mitochondrial polymerase gamma. Thus, the amount of mitochondrial polymerase gamma may be reduced during said treatment of HIV, which may result in a decreased amount of mitochondria per cell. A decrement of for instance 50% of the mitochondria may result in side-effects. The ratio of mitochondrial DNA versus nuclear DNA may be diminished by a factor 2. However, a decrement of mitochondrial DNA by a factor 2 can in some cases lie within the double spreading of the measurement of said ratio because of the mentioned variations in conditions. Therefore this biologically important difference in amount of mitochondria may not reliably be detected because of

double spreading in the result. Thus, double spreading can in some cases reduce the reliability of detection of biologically important differences in a ratio of nucleic acids and/or their gene products. Therefore, one embodiment of the present invention provides a method for determining functioning of a cellular organism, without double spreading in the result, comprising determining the relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from said organism in relation to the amount of a second nucleic acid and/or gene product thereof. Said double spreading can in a preferred embodiment of the present invention be prevented by determination of said ratio in the same assay. This means that a processing step and/or a measurement of the amounts of at least 2 nucleic acids and/or gene products thereof is performed in the same assay. In terms of the invention, an assay typically utilises one reaction mixture. Preferably, all components of an assay of the invention are mixed randomly in said assay. Said reaction mixture may be present in one reaction tube.

However, a person skilled in the art can think of more methods to prevent double spreading in the result. He/she can for instance use a reaction vessel which is divided in different parts by a (semi)permeable membrane. As long as at least one reaction condition varies dependently in said different parts, double spreading is avoided and the obtained result will be more accurate.

In one embodiment of the current invention at least two target sequences are amplified in one assay. Said two target sequences may be said endosymbiont cellular organelle nucleic acid and said second nucleic acid. Thus in one embodiment of the current invention a method of the invention is provided, comprising amplification of said endosymbiont cellular organelle nucleic acid and said second nucleic acid in the same assay. When at least two target sequences are amplified in one assay, varieties in reaction conditions in said assay can influence the obtained amount of each sequence present in said

assay dependently. For instance, the obtained amount of each sequence present in said assay will be influenced by the same temperature, the same overall volume, and so on. Detection of said two target sequences can be achieved by using two specific probes during the generation of amplified
5 nucleic acids during an amplification reaction. Said two probes may each have a different label allowing discrimination between said two probes and thereby between said two different target sequences. Quantification can be achieved by relating the time to positivity as well as the slope of the relative fluorescence increase of both real time amplification reactions. Thus there is no need for an
10 internal standard like for instance a competitor molecule. A method of relative quantification of two targets in one assay has an improved accuracy compared to quantification in two separate assays, and requires less handling time and reagents. We found that duplexing of two amplification reactions in the same tube gives an immediate indication of the ratio of the two targets. The
15 conditions of both amplification reactions are the same, ruling out variations of those conditions without the necessity for internal or external calibrators. Hence, double spreading in the result is now avoided. Thus, in one aspect the invention provides a method, wherein a relative ratio is determined directly by dividing one amount of nucleic acid by another. In terms of the invention,
20 determined directly means that an immediate indication of the ratio of the two targets is possible, for instance by comparing the intensity of said two different fluorescent labels of said two specific probes. In this embodiment, dividing one amount of nucleic acid by another is performed by dividing the intensity of the corresponding fluorescent label by another. No internal standards are used in
25 a method of the invention wherein said relative ratio is determined directly.

A method of the invention is especially useful to quantify a target nucleic acid and/or gene product thereof with a variable content in relation to a target nucleic acid and/or gene product thereof with a constant content. An
30 example is the quantification of the variable cellular content of mitochondrial

DNA to the constant cellular content of the DNA of a nuclear gene (two per diploid cell). Another example comprises the quantification of variably expressed RNA like mitochondrial RNA to constitutively expressed RNA that is essential for cell survival like the SNRNP U1A encoding RNA involved in
5 splicing or other essentially common nucleic acids derived from nuclear DNA with an ubiquitous presence. We found that it is possible to determine a relative ratio of a factor 2 à 3.

Furthermore, the invention provides a diagnostic kit comprising at least
10 one means for performing a method according to the invention, said kit comprising at least one primer or probe set selective for the amplification and detection of a nucleic acid related to or derived from endosymbiont cellular organelles and, when so desired, necessary amplification reagents, such as can be found exemplified in the detailed description herein or which are otherwise
15 known in the art. In particular, the invention provides a diagnostic kit wherein said kit comprises more than one primer or probe set for the amplification of nucleic acid sequences related to cellular organelles, preferably supplemented with a primer or probe set for the amplification of nucleic acid related to the chromosomes, such as a SNRNP specific primer or
20 probe. In particular the invention provides a kit comprising at least one primer or probe from table 1 for the amplification of nucleic acid sequences related to cellular organelles. It is of course preferred that said amplification reagents, when provided with the kit, comprise an enzyme with reverse transcriptase activity, such as required for PCR or NASBA amplification. Of course, a kit
25 comprising a means for the detection of a gene product other than nucleic acid, for use in a method according to the invention is herewith also provided.

The invention furthermore provides the use of a compound obtainable or detectable by a method according to the invention in the preparation of a medicament, a herbicide, insecticide, anti-parasiticum, cytostatic, etc, and a

medicament, herbicide, insecticide, anti-parasiticum etc. obtainable or derivable or identifiable by a method according to the invention.

5 The invention is further explained in the detailed description herein, wherein most examples are directed by way of example at testing of mitochondriae, being central to the provision and use of energy in a cell, however, it will easily be understood that the same principles apply to tests using other endosymbiont organelles, such as chloroplasts, being central to the provision of carbohydrates to a plant cell.

Examples

Used ingredients and general methodology

In table 1 the primers and probes used in the examples are summarised.

- 5 Standard NASBA nucleic acid amplification reactions were performed in a 20µl reaction volume and contained: 40mM Tris-pH 8.5, 70mM KCl, 12mM MgCl₂, 5mM dithiotreitol, 1mM dNTP's (each), 2mM rNTP's (each), 0.2µM primer (each), 0.05µM molecular beacon, 375mM sorbitol, 0.105 µg/µl bovine serum albumin, 6.4 units AMV RT, 32 units T7 RNA polymerase, 0.08 units
- 10 RNase H and input nucleic acid. The complete mixture, except the enzymes, sorbitol and/or bovine serum albumin was, prior to adding the enzyme mixture, heated to 65°C for 2 minutes in order to denature any secondary structure in the RNA and to allow the primers to anneal. After cooling the mixture to 41°C the enzymes were added. The amplification took place at 41°C
- 15 for 90 min in a fluorimeter (CytoFluor 2000) and the fluorescent signal was measured every minute (using the filter set 530/25 nm and 485/30 nm). For amplification of DNA target sequences the 65°C denaturation step was replaced with a 95°C denaturation step for the same period of time.
- To achieve quantification, a dilution series of target sequence for a particular
- 20 primer set was amplified and the time points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of nucleic acid. This way a calibration curve was created that could be used to read TTP values of reactions with unknown amounts of input and deduce the input amount. Examples of typical standard curves for quantification of RNA
- 25 and DNA are shown in figure 1.

For some of the target sequences no dilution series were available with reliable absolute amount of copies determined. Those series were given an arbitrary unit as measurement instead of DNA or RNA copies, e.g. cell-equivalent or ET-

unit. As a result it sometimes seems that there is less RNA than DNA, which is quite the opposite of what is expected.

Cells (fibroblasts and PBMC's) were cultured under standard conditions in standard media known to persons skilled in the art with addition of drugs or
5 putative toxic or stimulating compounds as defined in the examples. Nucleic acids were isolated from the cells with the method described by Boom et al (Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J, 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol*; 28(3):495-503) or with dedicated isolation kits purchased from
10 Qiagen (Qiagen GmbH, Max Volmer Strasse 4, 40724 Hilden, Germany) and used according to the manufacturer's protocols. A small aliquot of the isolated nucleic acid was analysed on an agarose gel and the remainder stored at -80°C until further analysis. Usually the nucleic acid was diluted 10 times with water and of the diluted nucleic acid usually 5 µl was used as input in the
15 NASBA amplification reactions.

Example 1

In this example it is explained what kind of ratio's can be measured with a method according to the invention and the meaning they can have in
20 diagnostic sense:

The invention for example provides determining the relative ratio of organelle DNA to chromosomal DNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of organelles per cell. Also is provided determining the ratio of organelle RNA to
25 chromosome encoded RNA. This ratio when compared with normal values or determined at at least two points in time, shows the organelle transcription activity decline or increase per cell, normalised for the active state (i.e. transcription state) of the cell.

Also is provided determining the ratio of organelle RNA to chromosomal DNA. This ratio when compared with normal values or determined at at least two points in time, shows the organelle transcription activity decline or increase per cell

- 5 Also provided is determining the ratio of organelle DNA to organelle RNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of transcription in the organelle, indicating regulation at the transcriptional level to achieve a certain mRNA (and therefore protein) level.
- 10 Also provided is determining the ratio of organelle DNA to chromosome encoded RNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of transcription in the cell, in relation to chromosomal RNA transcription levels, indicating the activity state of the organelle, which is especially useful when chromosomal
- 15 RNA is determined that encodes an organelle protein or other component thereof.

Example 2

- Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs
- 20 DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the
 - 25 cells was harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol. The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial DNA to chromosomal DNA ratio in the

culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial DNA content as expected. The results for DDC are shown in figure 2.

5 The data in figure 2 clearly show a decline in the amount of mitochondrial DNA per cell with more than 2 logs and therewith the mitochondrial toxicity of the antiviral drug DDC.

Example 3

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs
10 DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the
15 cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol. The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to chromosome encoded RNA ratio
20 in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA content as expected. The results for DDC are shown in figure 3. The data in figure 3 clearly show a decline in the amount of mitochondrial RNA per cell with at least 2 logs and therewith the mitochondrial toxicity of the antiviral drug DDC. The time point at 3 weeks
25 has a very low value and presumably this is somewhat of an outlier measurement.

Example 4

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol.

The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to chromosomal DNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA content as expected. The results for DDC are shown in figure 4.

The data in figure 4 clearly show a decline in the amount of mitochondrial RNA per cell with almost 3 logs and therewith the mitochondrial toxicity of the antiviral drug DDC. The time point at 3 weeks has a very low value and it presumably this is somewhat of a outlier measurement.

Example 5

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers

MtR p1 and MtR p2 and probe MtR mb) and mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) in the described NASBA protocol.

The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to mitochondrial DNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA and DNA content as expected. The results for DDC are shown in figure 5.

The data in figure 5 clearly show that the ratio of mitochondrial DNA to RNA in not significantly changing over the period of 4 weeks. The time point at 3 weeks in figure 5 has a low value for mitochondrial RNA that shows up, this measurement is presumably somewhat of an outlier measurement.

Example 6

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one-week intervals part of the cells was harvested and analyzed for amount of chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol.

The cultures with AZT, D4T, ethidium bromide and without additive showed no measurable change in ratio in the culture period of 4 weeks. The results for DDC are shown in figure 6.

The data in figure 6 clearly show that the ratio of chromosomal DNA to RNA in not significantly changing over the period of 4 weeks.

Example 7

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drug DDC at a concentration of 30 μ M for 4 weeks. After that period the cell culture continued but now in the absence of DDC. During this period of culture without DDC part of the cells was harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol at two-week intervals for a period of 12 weeks. The results of the analysis are shown in figure 7.

- 10 The results in figure 7 clearly show that the amount of mitochondria per cell increases with more than 2 logs after DDC is removed from the culture. This results shows that the toxic effect of DDC can be reversed if there are still some mitochondria left in the cells to repopulate the new growing cells.

Example 8

Fibroblast cells were cultured *in vitro* in the presence of the anti viral drug DDC at a concentration of 30 μ M for 4 weeks. After that period the cell culture continued but now in the absence of DDC. During this period of culture without DDC part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol at two-week intervals for a period of 12 weeks. The results of the analysis are shown in figure 8.

- 20 The results in figure 8 clearly show that the amount of mitochondrial RNA per cell increases with more than 2 logs after DDC is removed from the culture. This results shows that the toxic effect of DDC can be reversed and that the function of the mitochondria comes back as shown by synthesis of RNA and subsequently proteins.

Example 9

Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 6 μ M and 60 μ M, respectively, for 5 days. As
 5 controls cell cultures with DMSO and without drugs were also performed. DMSO is part of the solvent in which the drugs are solubelized. After 5 days the cells were harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described
 10 NASBA protocol.

The cultures with AZT, D4T, DMSO and without additive showed no measurable change in ratio in the culture period 5 days. The results for DDC are shown in figure 9.

The results in figure 9 clearly show the decline in PBMC's of mitochondrial
 15 DNA per cell of more than 1 log during the 5 day culture period.

Example 10

Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and
 20 D4T at two concentrations each, 6 μ M and 60 μ M, respectively, for 5 days. As controls cell cultures with DMSO and without drugs were also performed. DMSO is part of the solvent in which the drugs are solubelized. After 5 days the cells were harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded
 25 RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol.

The cultures with AZT, D4T, DMSO and without additive showed no measurable change in ratio in the culture period 5 days. The results for DDC are shown in figure 10. Interestingly, the results in figure 10 do not clearly

show a decline in PBMC's of mitochondrial RNA per cell during the 5-day culture period at the highest concentration of DDC used. This is in contrast to the mitochondrial DNA as shown in example 9. It is hypothesized that the decline in mitochondrial DNA is compensated by an increase in transcription, maintaining the level of mitochondrial RNA. This mechanism delays the decline of mitochondrial RNA.

Consequently, one can say that the mitochondrial RNA is a reflection of the current status of the functionality of the mitochondria and that mitochondrial DNA is predictive of what will happen in the (near) future with the mitochondrial function and therefore has a more prognostic character.

Example 11

Using the primers and probes Rubisco-DNA p1, Rubisco-DNA p2, Rubisco-DNA MB, Rubisco-RNA p1, Rubisco-RNA p2 and Rubisco-RNA-MB (table 1) the chloroplast DNA and RNA of *Oryza sativum* (rice) can be quantified and the ratio to the chromosomal DNA and RNA can be determined by using primers and probes OryzaDNA p1, OryzaDNA p2, OryzaDNA mb, OryzaRNA p1, OryzaRNA p2, OryzaRNA mb (table 1). During the application of herbicide (or other) compounds the conditions of the plants can be assessed by measurement of the chloroplast nucleic acid content of the cells using amplification methods like PCR and NASBA that are known to persons skilled in the art. At the same time, using primer sets suitable for weeds, the deterioration of the unwanted plants can be monitored. It is clear that these molecular tools are very suited in the research for new herbicides that specifically attack one group of plants and not others.

Example 12

In this example the NASBA nucleic acid amplification reactions for DNA target sequences were performed in a 20 μ l reaction volume and contained:

40mM Tris-pH 8.5, 70mM KCl, 12mM MgCl₂, 5mM dithiotreitol, 1mM dNTP's (each), 2mM rNTP's (each), 0.2μM primer (each), 0.05μM molecular beacon, 1.5 units restriction enzyme Msp I, 375mM sorbitol, 0.105 μg/μl bovine serum albumin, 6.4 units AMV RT, 32 units T7 RNA polymerase, 0.08 units RNase H
5 and input nucleic acid. The complete mixture, except the enzymes, sorbitol and bovine serum albumin was, prior to adding the enzyme mixture, incubated at 37°C for 25 minutes and subsequently heated to 95°C for two minutes in order to denature the DNA and to allow the primers to anneal. After cooling the mixture to 41°C the enzyme mixture was added. The amplification took place
10 at 41°C for 90 min in a fluorimeter (CytoFluor 2000) and the fluorescent signal was measured every minute (using the filter set 530/25 nm and 485/30 nm). To achieve quantification, a dilution series of target sequence for a particular primer set was amplified and the time points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of
15 nucleic acid. This way a calibration curve was created that could be used to read TTP values of reactions with unknown amounts of input and deduce the input amount. Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* for 5 days. After 5 days the cells were harvested and analyzed for amount of chromosomal DNA (primers SnrpD
20 p1 and SnrpD2 p2 and probe SnrpD mb) with the described NASBA protocol in the chapter "Used ingredients and general methodology" and compared with the NASBA protocol as described in this example. As can be clearly seen in figure 11 the DNA NASBA reactions with pre-treatment of restriction enzyme perform much better than without. The rationale for this observation is the
25 direct extension from the Msp I created 3' over the T7 promoter part of the p1 primer.

Example 13

Using the primers and probes tRNA-L-D p1, tRNA-L-D p2, tRNA-L-D MB, petB RNA p1, petB RNA p2 and petB RNA MB (table 1) the chloroplast DNA and RNA of *Oryza sativum* (rice) can be quantified and the ratio to the chromosomal DNA and RNA can be determined by using primers and probes OryzaDNA p1, OryzaDNA p2, OryzaDNA mb, OryzaRNA p1, OryzaRNA p2, OryzaRNA mb (table 1). During the application of herbicide (or other) compounds the conditions of the plants can be assessed by measurement of the chloroplast nucleic acid content of the cells using amplification methods like PCR and NASBA that are known to persons skilled in the art. At the same time, using primer sets suitable for weeds, the deterioration of the unwanted plants can be monitored. It is clear that these molecular tools are very suited in the research for new herbicides that specifically attack one group of plants and not others.

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Example 14

Thousand molecules of plasmid containing Snrp DNA were mixed with 4×10^5 , 2×10^5 , 10^5 , 5×10^4 , 2.5×10^4 , or 10^4 molecules of plasmid containing mitochondrial DNA, and the mixture was used as input for the reactions. A reaction mix was prepared similar to that of example 12, except that primers and beacons differed in order to amplify Snrp-nuclear and mitochondrial DNA in one tube. The reaction mix (duplex-mix) contained two sets of primers and beacon: SnrpD p1 and SnrpD p2, and MtD p1_2 and MtD p2_2 (each $0.2 \mu\text{M}$) with beacons SnrpD mb (ROX-labeled) and MtD mb_2 (FAM-labeled) (each $0.05 \mu\text{M}$). Restriction enzyme digestion, amplification, and detection were performed as in example 12. Filter sets of the fluorimeter (CytoFluor 2000) were adapted to simultaneously measure the FAM and the ROX-label (485/20 and 530/25 for FAM; 590/20 and 645/40 for ROX). In a duplex reaction with two competing amplifications the ratio of the slope of the curves of fluorescence

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in time is proportional to the ratio of the amount of molecules of each amplified species.

Example 15

5 PBMC were cultured in the absence and presence of 5 μ M ddC. After 5 days PBMC samples were drawn. Nucleic acids were isolated from 10^5 PBMC according to the method described by Boom et al. and dissolved in 50 μ l DNase and RNase free water. A 1:10 and 1:100 dilution was made, and 5 μ l of the dilution (equivalent to 1,000 or 100 PBMC, respectively) was put in the
10 reaction mix to amplify the specific targets. In parallel, 10^3 molecules of plasmid containing Snrp DNA was mixed with 4×10^5 , 2×10^5 , 10^5 , or 5×10^4 molecules of plasmid containing mitochondrial DNA, and the mixture was used as input for the reactions. A reaction mix was prepared similar to that of example 12, except that primers and beacons differed in order to amplify Snrp-
15 nuclear and mitochondrial DNA in one tube. The reaction mix (duplex-mix) contained two sets of primers and beacon: SnrpD p1 and SnrpD p2, and MtD p1_2 and MtD p2_2 (each 0.2 μ M) with beacons SnrpD mb (ROX-labeled) and MtD mb_2 (FAM-labeled) (each 0.05 μ M). Restriction enzyme digestion, amplification, and detection were performed as in example 12. Filter sets of
20 the fluorimeter (CytoFluor 2000) were adapted to simultaneously measure the FAM and the ROX-label (485/20 and 530/25 for FAM; 590/20 and 645/40 for ROX). In a duplex reaction with two competing amplifications the ratio of the slope of the curves of fluorescence in time is proportional to the ratio of the amount of molecules of each amplified species. The data of the plasmid Snrp/
25 mitochondrial DNA mixtures were used to create a standard curve on which the unknown ratio of mitochondrial to Snrp nuclear DNA of the PBMC samples in the dilutions 1:10 and 1:100 in the absence and presence of 5 μ M ddC could be assessed.

Table 1. Sequences of primers and probes used in the examples.

Name	Sequence ¹
MtD p1	5' AATTCTAATACGACTCACTATAGGGAGAAGAGCCGTTGAGTTGTGGTA 3'
MtD p2	5' TCTCCATCTATTGATGAGGGTCTTA 3'
MtD mb	5' GCATGCCCCCTCCTAGCCTTACTACTAATGCATGC
MtD p1_2	AAT TCT AAT ACG ACT CAC TAT AGG GAA GAA CCG GGC TCT GCC ATC TTA A
MtD p2_2	GTA ATC CAG GTC GGT TTC TA
MtD mb_2	GGA CCC CCC ACA CCC ACC CAA GAA CAG GGT CC
SnrpD p1	5' AATTCTAATACGACTCACTATAGGGAGAGGCCCGGCATGTGGTGCATAA 3'
SnrpD p2	5' TTCCTTACATCTCTCACCCGCTA 3'
SnrpD mb	5' GCATGCTGTAACCACGCACTCTCCTCGCATGC 3'
SnrpD2 p2	5' TGCGCCTCTTTCTGGGTGTT 3'
MtR p1	5' AATTCTAATACGACTCACTATAGGGAGGAGAAGATGGTTAGGTCTAC 3'
MtR p2	5' CGATATGGCGTTCCCCCGCATAAA 3'
MtR mb	5' GCTCCG AAGCTTCTGACTCTTACCTCCC CGGAGC 3'
MtR p1_2	AAT TCT AAT ACG ACT CAC TAT AGG GAG AGG AGA CAC CTG CTA GGT GT
MtR p1_3	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GGT AGA CTG TTC AAC CTG TT
MtR p2_2	GGT GCC CCC GAT ATG GCG TTC C
MtR p2_3	GTA ATA ATC TTC TTC ATA GTA A
SnrpR p1	5' AATTCTAATACGACTCACTATAGGG AGAGGCCCGGCATGTGGTGCATAA 3'
SnrpR p2	5' CAGTATGCCAAGACCGACTCAGA 3'
SnrpR mb	5' CGTACGAGAAGAGGAAGCCCAAGAGCCACGTACG 3'
SnrnpR p1_2	AAT TCT AAT ACG ACT CAC TAT AGG G A GAA GAA GAT GAC AAA GGC CTG GCC
SnrnpR p1_3	AAT TCT AAT ACG ACT CAC TAT AGG G A GAA AAA GGC CTG GCC CCT CAT CTT
SnrnpR p2_2	TCC ATG GCA GTT CCC GAG A
SnrnpR p2_3	CAC TAT TTA TAT CAA CAA CC
SnrnpR p2_4	TCA ATG AGA AGA TCA AGA A
SnrnpR mb_2	CGA TCG AGT CCC TGT ACG CCA TCT TC CGA TCG
Rubisco-DNA p1	5' AATTCTAATACGACTCACTATAGGGGGATAATTTTCATTACCTTCACGAG 3'
Rubisco-DNA p2	5' GGAGTCTGAACTAGCCGAG 3'
Rubisco-DNA MB	5' GCATGCGGTAGATAAACTAGATAGCTAGGCATGC 3'
Rubisco-RNA p1	5' AATTCTAATACGACTCACTATAGGGGAGTTGTTGTTATTGTAAGTC 3'
Rubisco-RNA p2	5' CAAGTCTTATGAATTCCTATAG 3'
Rubisco-RNA-MB	5' GCTAGCACACAGGGTGTACCCATTATGCTAGC 3'
OryzaDNA p1	5' AATTCTAATACGACTCACTATAGGGGGATCTTAATTACATGCCGTTCA 3'

OryzaDNA p2	5' AAAGGTGCCGGTTCTCACTA 3'
OryzaDNA mb	5' GCTAGCCTCTGCAAGCTTCATCAGTAATAGGCTAGC 3'
OryzaRNA p1	5' <i>AATTCTAATACGACTCACTATAGGGGCTAATGCCCTTTTCTTTCTTCCTC</i> 3'
OryzaRNA p2	5' CATATTGGCT TTCGAAGATT 3'
OryzaRNA mb	5' GCTAGCCTTCAGCCATTATTCAAGAT GGTGGCTAGC 3'
tRNA-L-D p1	5' <i>AATTCTAATACGACTCACTATAGGGGGGTTCTAGTTCGAGAACCGCTTG</i> 3'
tRNA-L-D p2	5' GCGAAATCGGTAGACGCTACG 3'
tRNA-L-D MB	5' GCTAGCCAACTTCCAAATTCAGAGAAGCTAGC 3'
petB RNA p1	5' <i>AATTCTAATACGACTCACTATAGGGAAACCGGTAGCAACTTGTACTAG</i> 3'
petB RNA p2	5' GGTTCGGTATCTCTGGAATATGAG 3'
petB RNA MB	5' GCTAGCGAGGAACGTCTTGAGATTCAGCTAGC 3'

1. The T7 promoter part of primer p1 sequences is shown in *italics*, the stem sequences of the molecular beacon probes are shown in **bold**. The molecular beacon sequences were labelled at the 3' end with DABCYL (the quencher) and at the 5' end with 6-FAM (the fluorescent label).

Brief description of the drawings

- 5 Figure 1. Examples of standard curves for DNA and RNA target sequences.
- Figure 2. Ratio of mitochondrial DNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.
- 10 Figure 3. Ratio of mitochondrial RNA and chromosome encode RNA in fibroblast cells cultured in the presence of DDC.
- Figure 4. Ratio of mitochondrial RNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.
- 15 Figure 5. Ratio of mitochondrial RNA and mitochondrial DNA in fibroblast cells cultured in the presence of DDC.
- Figure 6. Ratio of chromosome encoded RNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.
- 20 Figure 7. Ratio of mitochondrial DNA and chromosomal DNA in fibroblast cells cultured in the absence of DDC after being cultured with DDC for 4 weeks.
- 25 Figure 8. Ratio of mitochondrial RNA and chromosome encoded RNA in fibroblast cells cultured in the absence of DDC after being cultured with DDC for 4 weeks.
- 30 Figure 9. Ratio of mitochondrial DNA and chromosomal DNA in PBMC's cultured in the presence of DDC for 5 days.

Figure 10. Ratio of mitochondrial RNA and chromosome encoded RNA in PBMC's cultured in the presence of DDC for 5 days.

- 5 Figure 11. Comparison of SNRNP DNA NASBA reactions with and without pre-treatment with restriction enzyme Msp I.

Figure 12. Fluorescence in time of the reactions of 1000 molecules plasmid containing Snrp DNA mixed with 4×10^5 (A), 2×10^5 (B), 10^5 (C), 5×10^4 (D),
 10 2.5×10^4 (E) or 10^4 (F) molecules of plasmid containing mitochondrial DNA. The curve (G) of the ratio of the amount of molecules of amplified mitochondrial DNA to Snrp nuclear DNA plotted against ratio of the slope of the corresponding fluorescence in time

- 15 Figure 13. Fluorescence in time of the reactions of 1000 molecules plasmid containing Snrp DNA mixed with 4×10^5 (A), 2×10^5 (B), 10^5 (C), or 5×10^4 (D) molecules of plasmid containing mitochondrial DNA. The standard curve (E) of the ratio of the amount of molecules of amplified plasmid mitochondrial DNA to plasmid Snrp nuclear DNA plotted against ratio of the slope of the
 20 corresponding fluorescence in time as derived from the figures A-D; closed circles indicate data points. The 1:10 (F, H) and 1:100 (G,I) dilutions of PBMC in the absence (F, G) and presence of $5 \mu\text{M}$ ddC (H, I). In figure E, the squares represent the PBMC samples cultured in the absence of ddC and the diamonds represent PBMC samples cultured in the presence of $5 \mu\text{M}$ ddC.

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Claims

1. A method for determining functioning of a cellular organism comprising determining the relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from said organism in relation to the amount of a second
5 nucleic acid and/or gene product thereof.
2. A method according to claim 1 whereby said relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof is determined in relation to the amount of a nuclear nucleic acid and/or gene product thereof detectable in said sample.
- 10 3. A method according to claim 2 wherein said nuclear nucleic acid comprises DNA.
4. A method according to claim 3 wherein said nuclear nucleic acid comprises DNA encoding a component of a small nuclear ribonucleoprotein or fragment thereof.
- 15 5. A method according to claim 2 wherein said nuclear nucleic acid comprises RNA.
6. A method according to claim 5 wherein said nuclear nucleic acid comprises RNA encoding a component of a small nuclear ribonucleoprotein or fragment thereof.
- 20 7. A method according to anyone of claims 1 to 7 wherein said endosymbiont cellular organelle nucleic acid and/or or gene product thereof comprises RNA.
8. A method according to claim 1 whereby said relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product
25 thereof is determined in relation to the amount of a second endosymbiont cellular organelle nucleic acid and/or or gene product thereof detectable in said sample acid.

9. A method according to claim 8 wherein said first nucleic acid comprises DNA.
10. A method according to claim 8 wherein said first nucleic acid comprises RNA.
- 5 11. A method according to anyone of claims 1, 2 or 8 wherein said first nucleic acid comprises DNA and wherein said second nucleic acid comprises RNA.
12. A method according to claim 11 whereby said second nucleic acid is in essence derivable by transcription from said first nucleic acid.
- 10 13. A method for determining therapeutic activity and/or possible side-effects of a candidate compound in determining its usefulness for treatment of malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism.
- 15 14. A method according to claim 13, wherein said therapeutic activity comprises a therapeutic activity against an HIV-related disease.
15. A method according to claim 13 or 14 wherein said organism or an essentially related organism has been provided with said compound.
- 20 16. A method for determining toxic activity of a candidate compound in determining its usefulness for causing malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism.
- 25 17. A method according to claim 15 wherein said organism or an essentially related organism has been provided with said compound.
18. A method for determining selective activity of a candidate compound against a first organism comprising determining therapeutic activity and/or possible side-effects of a candidate compound with a method

according to claim 13 or 14 and/or determining toxic activity with a method according to claim 15 or 16.

19. A method according to claim 17 further comprising providing an essentially unrelated second organism with said compound.

5 20. A method according to claim 18 wherein said first organism comprises a pathogen and said second organisms comprises a host for said pathogen.

10 21. A method according to claim 18 wherein said first organism comprises a weed plant and said second organism comprises a crop plant.

22. A method according to anyone of claims 1-21, wherein said relative ratio is determined in the same assay.

15 23. A method according to claim 22, comprising amplification of said endosymbiont cellular organelle nucleic acid and said second nucleic acid in the same assay.

24. A method according to claim 22 or 23, wherein said relative ratio is determined directly by dividing one amount of nucleic acid by another.

20 25. A diagnostic kit comprising at least one means for performing a method according to anyone of claims 1 to 24.

26. A kit according to claim 25, comprising at least one primer or probe selective for the amplification and detection of a nucleic acid related to or derived from endosymbiont cellular organelles.

25 27. A kit according to claim 26 wherein said primer or probe is listed in Table 1.

28. Use of a compound obtainable or selectable by a method according to the invention in the preparation of a medicament, a herbicide, an insecticide, an anti-parasiticum, an cytostatic agent or cytotoxic agent.

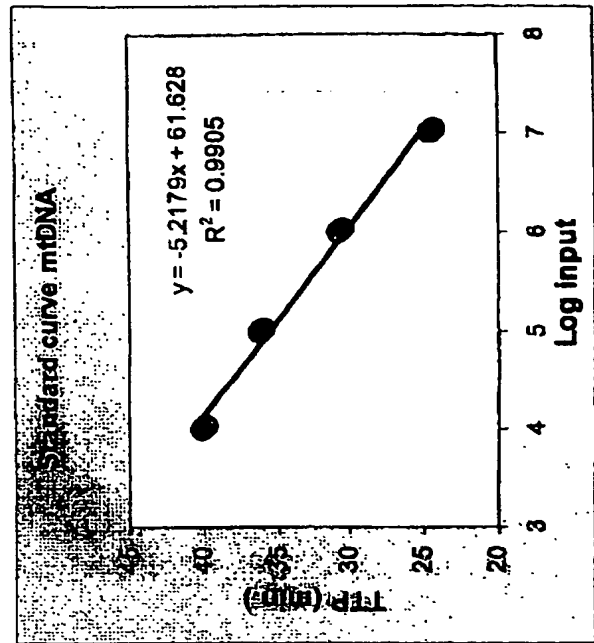
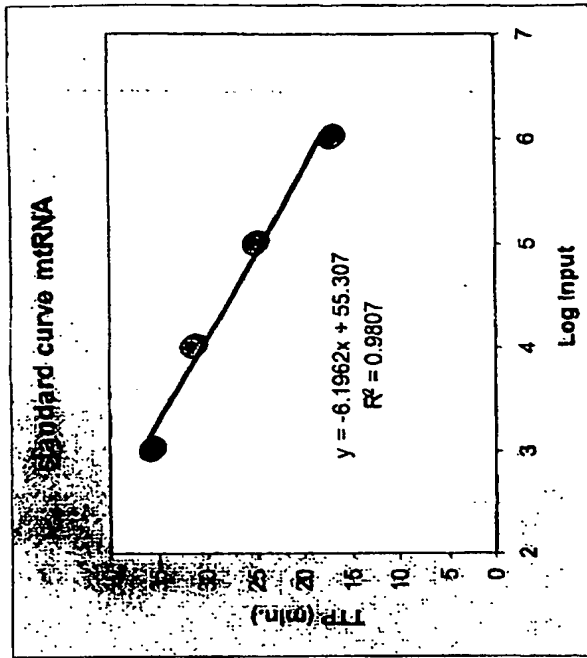
29. A medicament, a herbicide, an insecticide, an anti-parasiticum, an cytostatic agent or cytotoxic agent obtainable or selectable by a method according to the invention.

Abstract

The invention relates to the diagnosis of disease or the determination of functioning of cellular organisms, being of multi-cellular or unicellular nature, being visible by the naked eye or being a micro-organism. The invention provides a method for determining functioning of a cellular organism comprising determining the relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from said organism.

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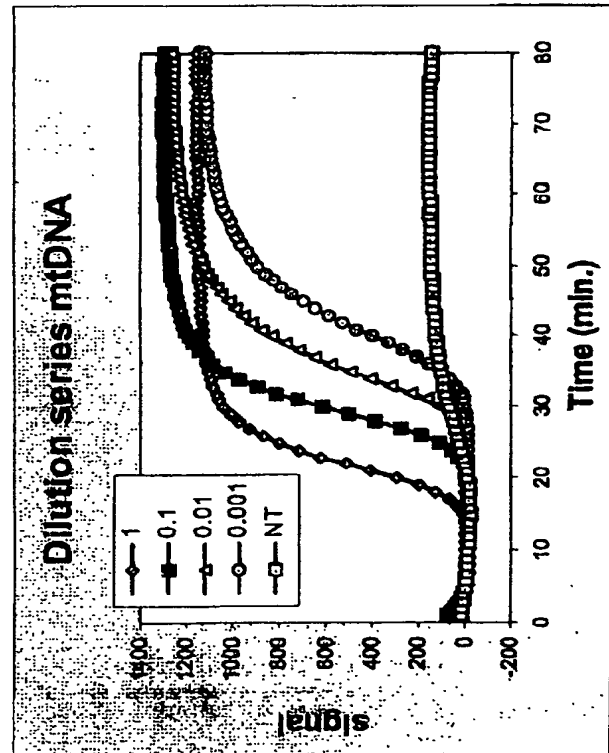
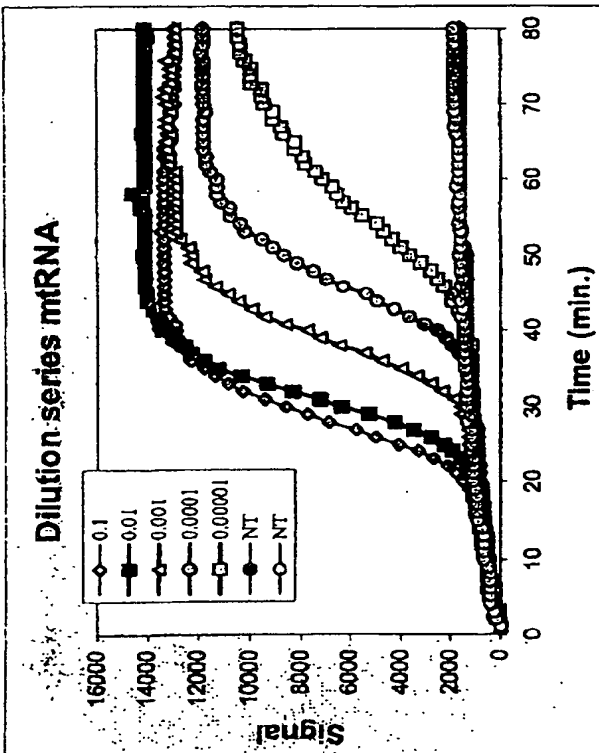


Figure 1

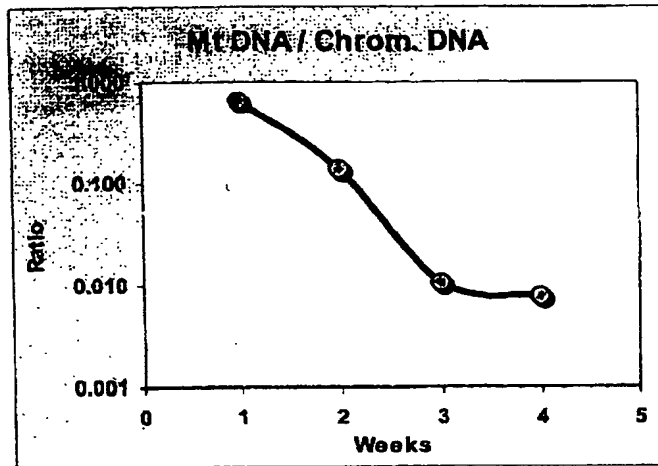


Figure 2

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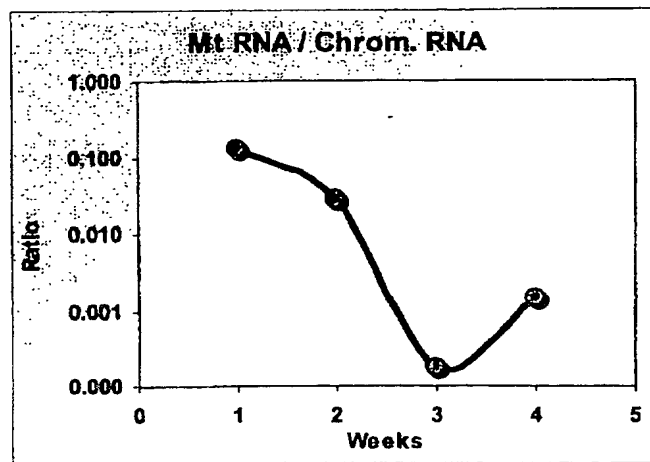


Figure 3

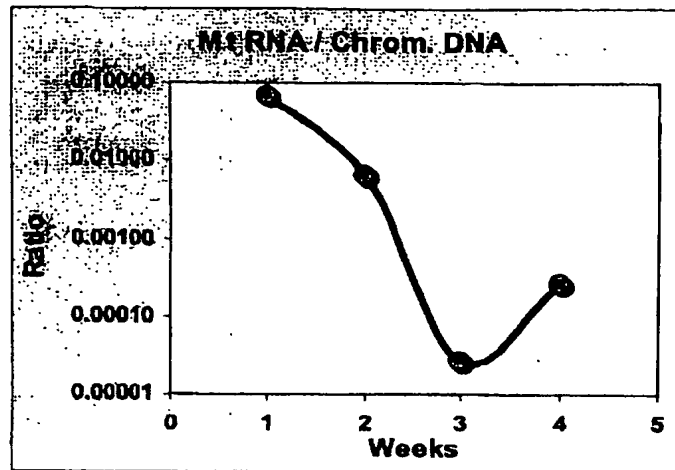


Figure 4

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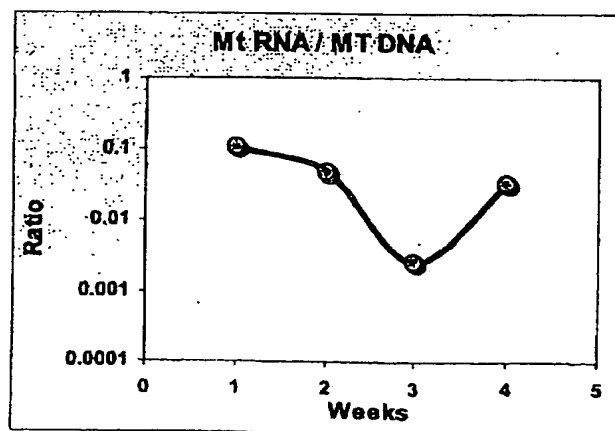


Figure 5

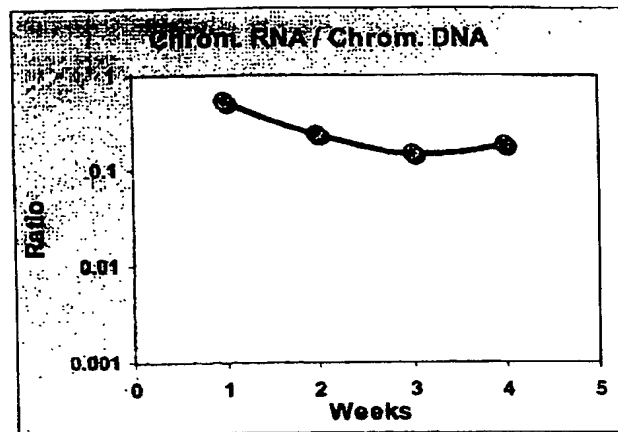


Figure 6

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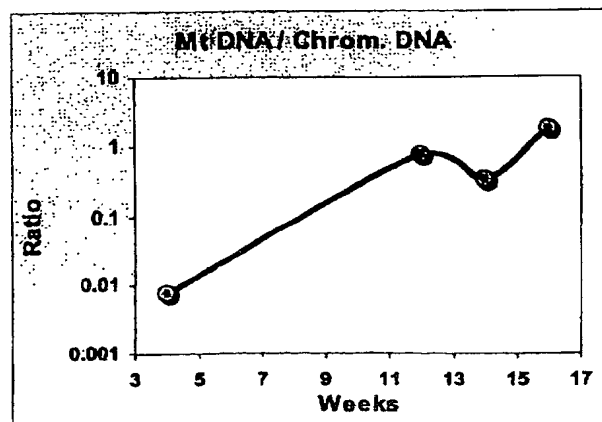


Figure 7

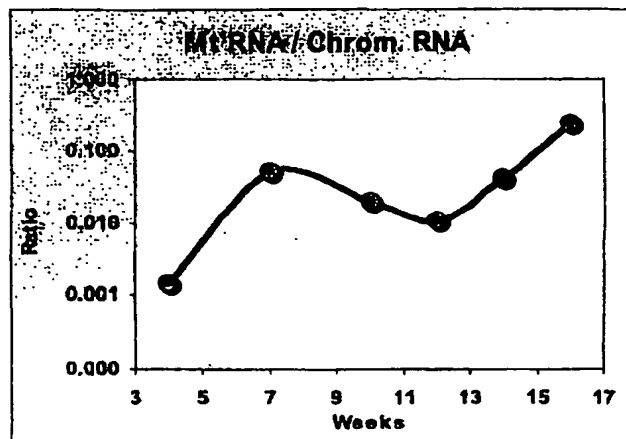


Figure 8

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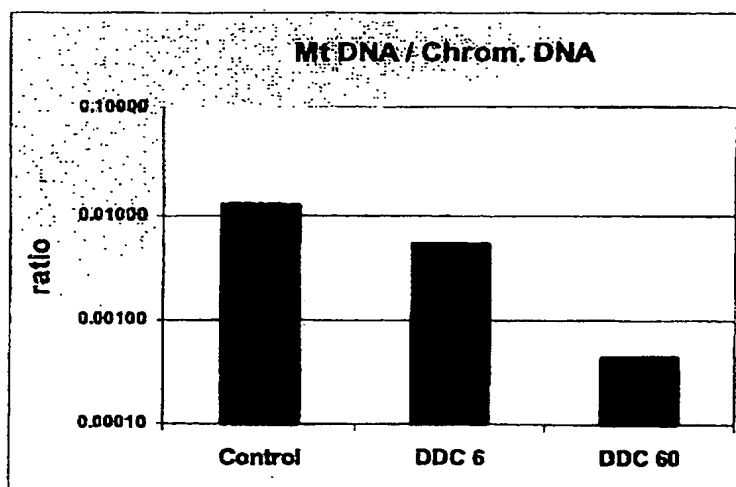
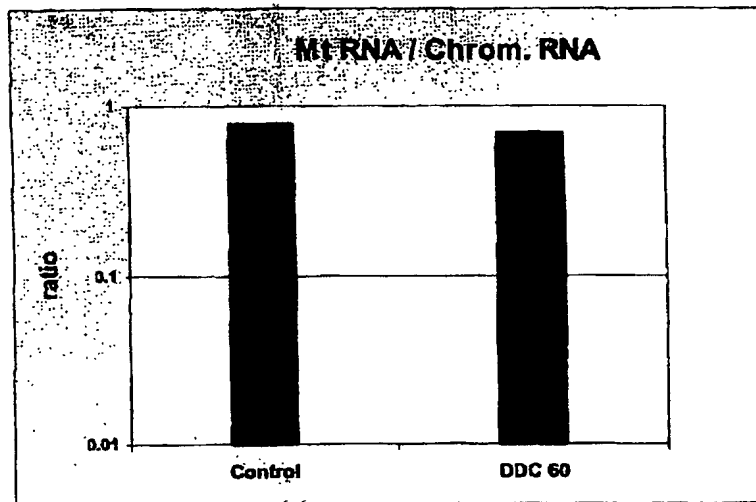


Figure 9

**Figure 10**

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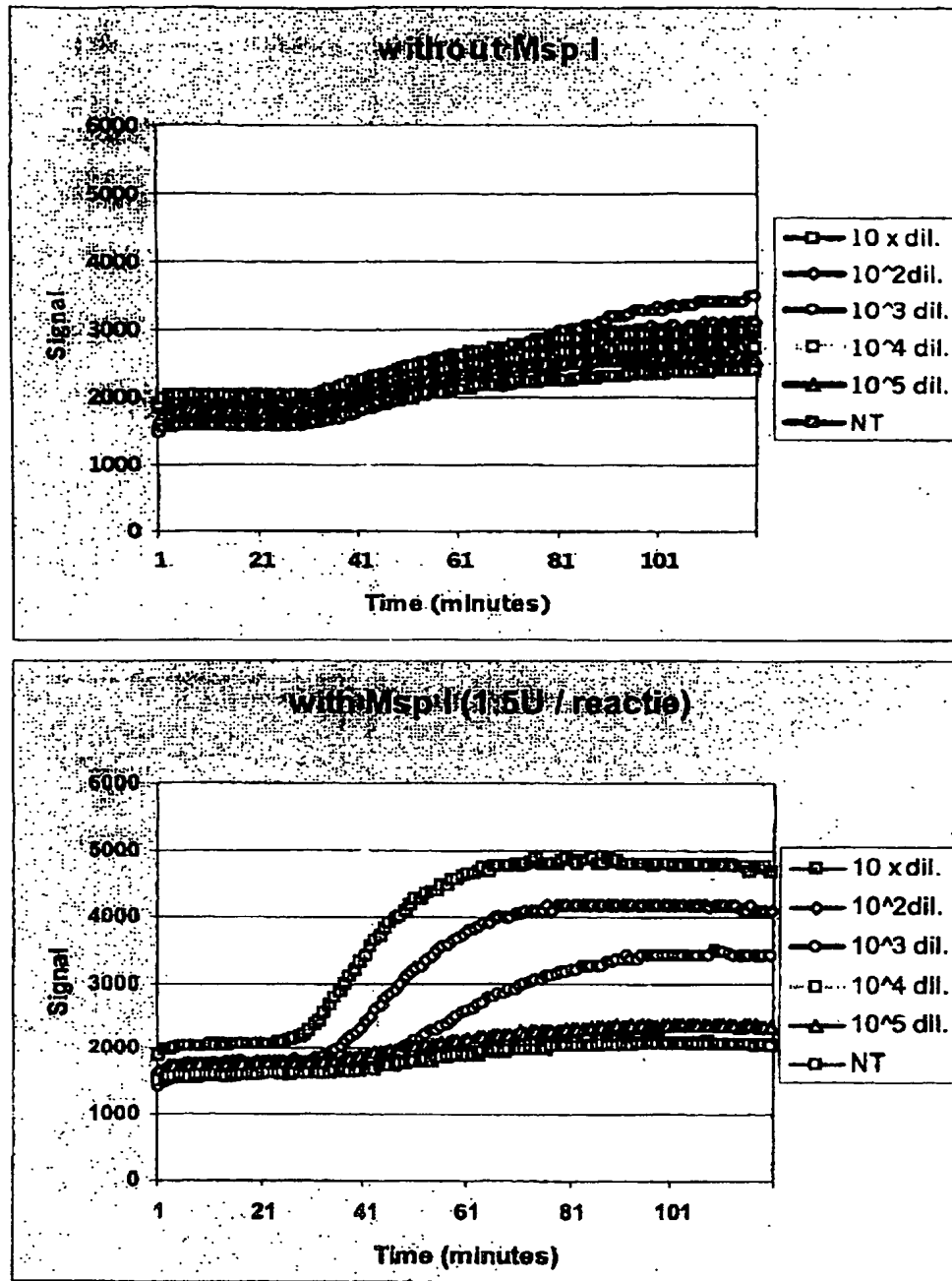
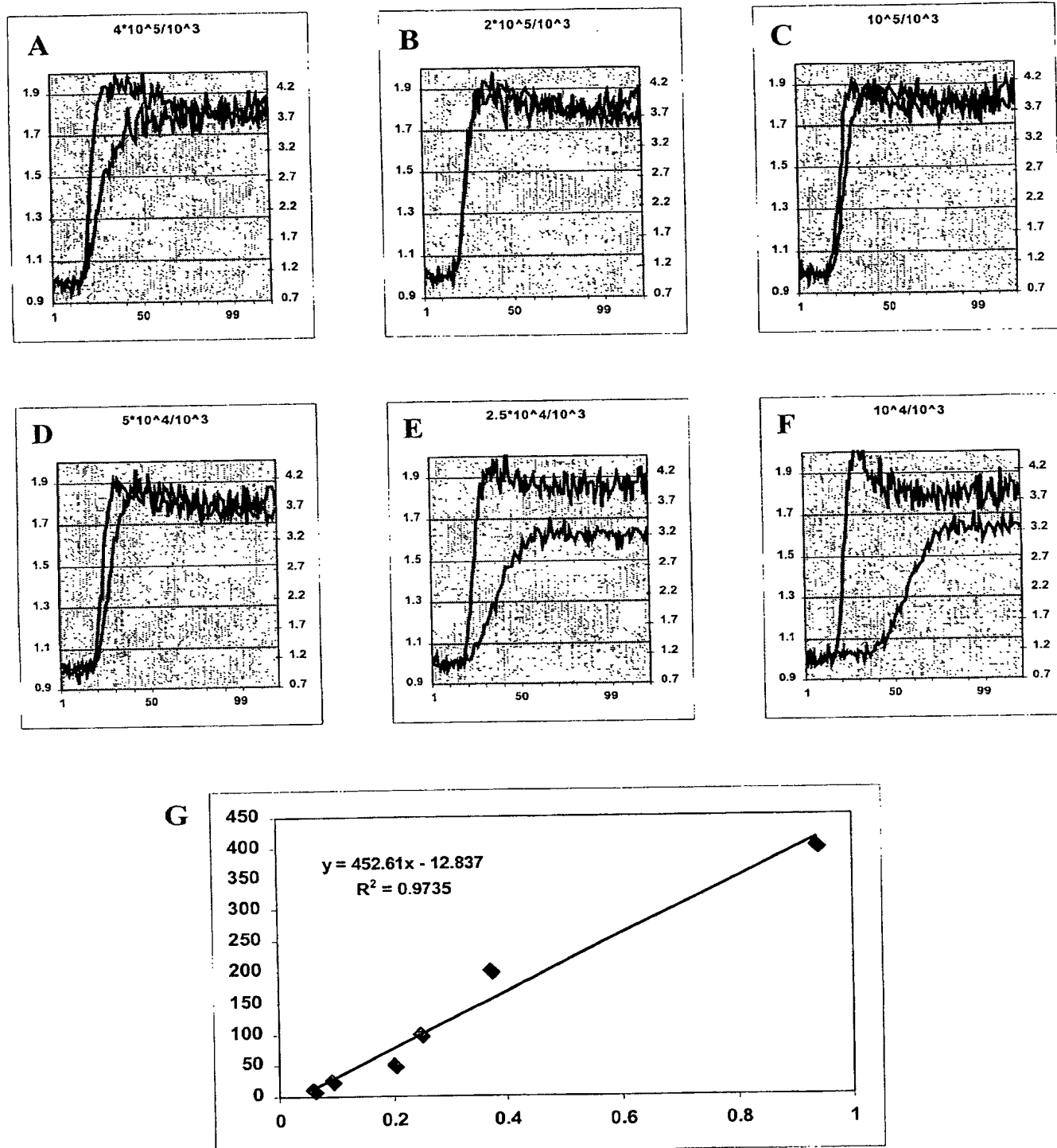


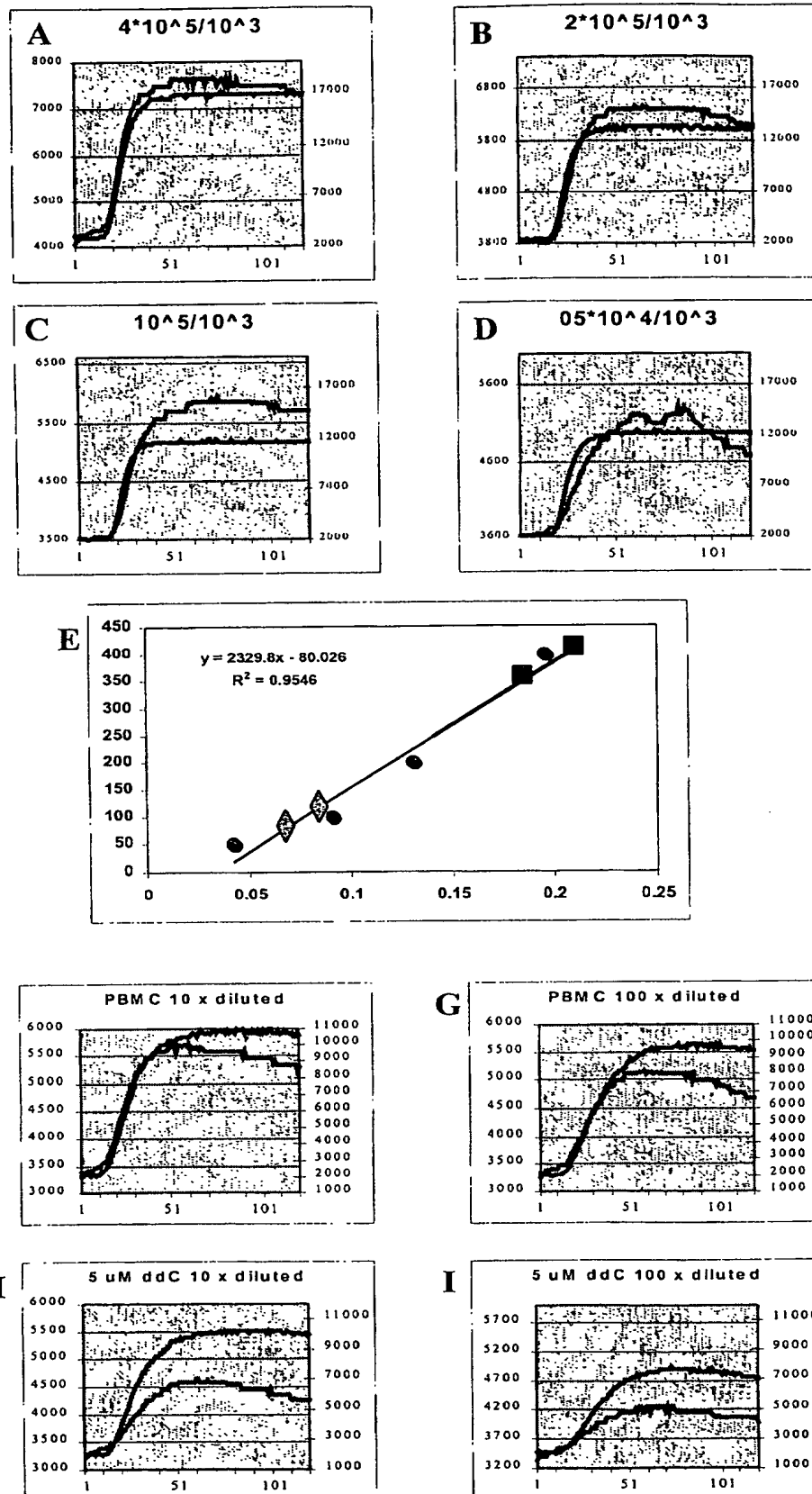
Figure 11. Comparison of Snrp DNA NASBA reactions with and without pre-treatment with restriction enzyme Msp I

Figure 12



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Figure 13



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